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Very-KIND is a novel nervous system specific guanine nucleotide exchange factor for Ras GTPases

Anaid Mees[a], Rebecca Rock[b], Francesca D. Ciccarelli[c,1], Cornelia B. Leberfinger[a], Johanna M. Borawski[a], Peer Bork[c], Stefan Wiese[d,2], Manfred Gessler[b,3], Eugen Kerkhoff[a,*]

[a]Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), Universität Würzburg, Versbacher Str. 5, 97078 Würzburg, Germany
[b]Theodor-Boveri-Institut (Biozentrum), Universität Würzburg, Physiologische Chemie I, Am Hubland, 97074 Würzburg, Germany
[c]European Molecular Biology Laboratory (EMBL), Meyerhofstr. 1, 69012 Heidelberg, Germany
[d]Institut für Klinische Neurobiologie, Josef-Schneider-Str. 11, 97080 Würzburg, Germany

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Abstract

The kinase non-catalytic c-lobe domain (KIND) evolved from the catalytic protein kinase fold into a potential protein interaction module for signalling proteins. Spir family actin organizers and the non-receptor phosphatase type 13 (PTP type 13) encode a KIND domain in the very N-terminal parts of the proteins. Here we report the characterization and cloning of a third member of the KIND protein family, which we have named very-KIND (VKIND) because of its two KIND domains. Like the other members of the protein family, VKIND has a KIND domain at the N-terminus. A second KIND domain is located in the central part of the protein. The C-terminal half encodes a guanine nucleotide exchange factor motif for Ras-like GTPases (RasGEF) and a RasGEF N-terminal module (RasGEFN). There is only one *VKIND* gene in the mammalian genomes and up to now we have found the gene only in vertebrates. During mouse embryogenesis the *VKIND* gene was specifically expressed in the developing nervous system. In adult mice Northern hybridizations revealed high expression only in brain. Low expression could be detected in ovary. In situ hybridizations showed a specific expression of *VKIND* in neuronal cells of the granular and Purkinje cell layers of the cerebellum.
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Keywords: Nervous system; Cerebellum; Signal transduction; Ras; RasGEF; KIND

1. Results and discussion

The kinase non-catalytic C-lobe domain (KIND) is a novel protein module, which was originally identified as a conserved domain contained in the N-terminal region of the Spir family actin organizers (Ciccarelli et al., 2003). The module evolved from the catalytical protein kinase fold into a potential protein interaction domain (Ciccarelli et al., 2003). The protein kinase fold harbours two structurally independent sub domains, the N-lobe, which is mainly formed by β-sheet and a larger and mainly helical C-lobe (Knighton et al., 1991). In between those lobes the catalytic cleft is located, where the ATP for the γ-phosphate transfer is bound. The KIND module only maintains the kinase C-lobe, losing the active residues both in the catalytic and activation sites (Ciccarelli et al., 2003). Within the family of kinases, the KIND bearing sequences are closely related to the family of p21 activated kinases (Paks). The C-lobe mediates the interaction between the kinases and the upstream activator proteins, the downstream phosphorylation targets or the regulatory subunits (Lei et al., 2000; Tanoue and Nishida, 2002). A possible function of the KIND domain could therefore, be the attraction of signalling proteins to KIND family proteins. Besides the Spir actin nucleation factors (Kerkhoff et al., 2001; Quinlan et al., 2005), data base searches identified the domain in two

* Corresponding author. Tel.: +49 931 201 45868; fax: +49 931 201 45835.
E-mail addresses: francesca.ciccarelli@embl.de (F.D. Ciccarelli), wiese_s@klinik.uni-wuerzburg.de (S. Wiese), gessler@biozentrum.uni-wuerzburg.de (M. Gessler), kerkhoff@mail.uni-wuerzburg.de (E. Kerkhoff).
[1] Tel.: +49 6221 387 8456; fax: 49 6221 387 8517.
[2] Tel.: +49 931 201 49789; fax: 49 931 201 49788.
[3] Tel.: +49 931 888 4159; fax: 49 931 888 7038.

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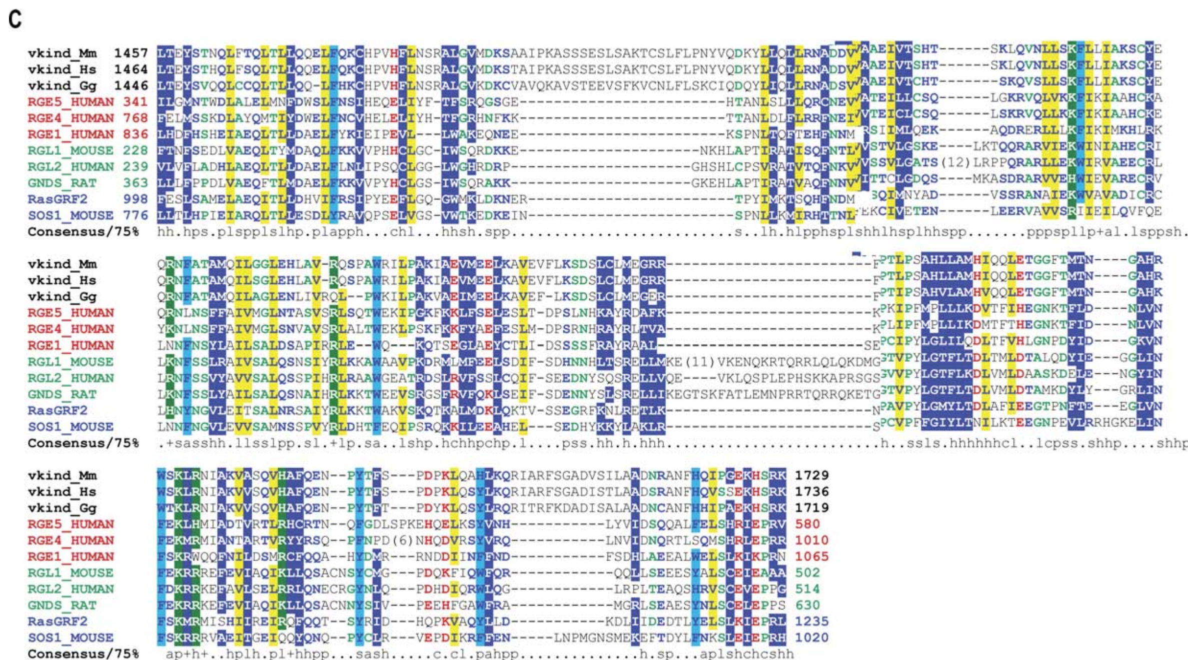
A

B

Fig. 1. Structure of the very-KIND protein. (A) Array of structural domains in the very-KIND protein. Two kinase non-catalytic C-lobe domains (KIND) are located in the N-terminal half of the protein. In the C-terminal part a guanine nucleotide exchange factor for Ras-like small GTPases (RasGEF) motif and a RasGEF N-terminal domain (RasGEFN) are located. (B) Alignment of human (*Homo sapiens*, hs), mouse (*Mus musculus*, Mm), chicken (*Gallus gallus*, Gg) and fish (*Fugu rubripes*, Fr) very-KIND protein sequences. The sequences of the KIND domains are underlined in pink, the ones of the RasGEFN and RasGEF domains are underlined in blue and green, respectively. Coloured letters within the sequence denote conserved amino acids. Cysteines are shown in pink letters, charged amino acids in red, small amino acids in green and polar amino acids in blue. Negative residues are highlighted in red, aliphatic in yellow, positive residues in green, aromatic in cyan and hydrophobic in blue. (C) Alignment of VKIND RasGEF domain to representatives of other guanine exchange factor domains for GTPases of the Ras subfamily. The Rap GEFs are represented in red, the Ral GEFs in green and the H-Ras GEFs in blue. For each domain, the starting and ending residues are reported. The average % sequence identity between the VKIND Ras GEF domains and the RasGEF domains of the other



(Fig. 1 *continued*)

additional protein families (Ciccarelli et al., 2003). One is the non-receptor tyrosine phosphatase type 13 (PTP type 13, PTP-Bas, PTP-BL) (Erdmann, 2003) and the other one is a protein sequence deduced from a human expressed sequence tag (EST) KIAA1768.

By characterizing the mouse ortholog of the human KIAA1768 EST clone (Accession: NP_689856), we show here that the third member of the KIND family is a guanine nucleotide exchange factor for Ras-like GTPases. Because of its two KIND domains it was designated very-KIND (VKIND). An open reading frame of 5229 base pairs has been deduced from sequenced cDNA fragments, which we generated by RT-PCR in analogy to the human KIAA1768 clone, and the partial sequence of a mouse gene, which was predicted by automated computational analysis (accession: XM_133920). The sequence was confirmed by DNA sequence analysis and a cDNA fragment covering the complete coding region was generated by cloning RT-PCR fragments, employing mouse cerebellum RNA as template (asseccion: AJ580324).

Analysis of the very-KIND protein sequence with the *Simple Modular Architecture Research Tool* (SMART) for identification and annotation of protein domains (Letunic et al., 2004; Schultz et al., 1998) revealed that the VKIND protein has two KIND domains in the N-terminal half, and a guanine nucleotide exchange factor domain for Ras-like small GTPases at the C-terminal end with a structural domain attached at its N-terminal (RasGEFN motif) (Fig. 1A). The Ras superfamily of small GTPases includes over 150 proteins (Ehrhardt et al., 2002). It has six subfamilies, the Ras, Rho, Ran, Rab, Arf and Kir/Rem/Rad subfamilies, which have roles in cancer, cell cycle regulation, differentiation, vesicle trafficking, control of cell morphology, chemotaxis and nuclear transport. The small GTPases cycle between an inactive GDP and an active GTP bound form. The activation mechanism requires guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP (Ehrhardt et al., 2002; Vetter and Wittinghofer, 2001). Despite the high structural conservation of the Ras superfamily proteins the GEF domains, which mediate the nucleotide exchange, are distinct for each subfamily. Rho subfamily GEFs have a DH (dibble-homology) catalytic domain (Schmidt and Hall, 2002). Exchange factors specific for Ras-subfamily GTPases have a catalytic domain with homology to the yeast RasGEF Cdc25 (Boriack-Sjodin et al., 1998; Ehrhardt et al., 2002). The very-KIND RasGEF domain is a Cdc25 like domain with highest homology to the RasGEF domains of exchange factors specific for Rap GTPases such as the Epac, MR-GEF and RA-GEF proteins (Fig. 1C).

The mouse sequence was used as a query for a tBLASTn search (Altschul et al., 1997) against the

families is 23% for RapGEFs, 20% to Ral GEFs and 19% to H-Ras GEFs. The residue colour scheme is the same as reported in Fig. 1B. The sequences have the following accession numbers: VKIND_Mm: CAE30489.2; VKIND_Hs: NP_689856.5; RGE5_Human: Q92565; RGE4_Human: Q8WZA2; RGE1_Q13905; RGL1_Mouse; Q60695; RGL2_Human: O15211; GNDS_RAT: Q03386; RasGRF2: NP_008840; SOS1_Mouse: Q62245. The VKIND sequence from *Gallus gallus* is a computional prediction and has not been released to the data bases. For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.

completed fully sequenced genomes of *R. norvegicus* (RGSC build 3.1), *F. rubripes* (Fugu build 2.0), *G. gallus* (WASHUC build 1), *C. elegans* (WS build 116), *D. melanogaster* (BGDP build 3.1). Putative orthologs were found only in vertebrates, but not in nematods and insects. The gene structure was refined using GeneWise (Birney et al., 2004). The predicted sequence in Fugu is not complete due to the presence of a gap region in the corresponding scaffold. A BLAST search was conducted in the EST non-mouse non-human database at NCBI using the mouse sequence as a query. EST evidences were found in *X. laevis*, *G. gallus*, *R. norvegicus* and the bird *T. guttata*. An alignment of the human, mouse, chicken and fugu protein sequences shows a high conservation within the predicted KIND, RasGEFN and RasGEF domains (Fig. 1B). In-between the second KIND domain and the RasGEFN domain two clusters of high conservation of yet unknown functional and structural identity are located (Fig. 1B).

Within the family of Cdc25 like gunanine nucleotide exchange factors the VKIND catalytic domain is closest related to RasGEFs specific for Rap GTPases with an average sequence identity between the VKIND RasGEF domains and the Rap specific RasGEF domains of 23% (Fig. 1C). A sequence alignment of the VKIND RasGEF domains with the enzymatic domains of RasGEF specific for Rap, Ral and Ras GTPases revealed that the VIKND RasGEF domains have an unique structure, in that they have two insertions, one of 24 amino acids in the N-terminal part of the domain (between helixes αA and αB of the Sos1 RasGEF structure, (Boriack-Sjodin et al., 1998)) and one of 11 amino acids in the C-terminal part (between helixes αJ and αK of the Sos1 RasGEF structure, (Boriack-Sjodin et al., 1998)) (Fig. 1C).

The mouse very-KIND protein contains 1742 amino acids and has a calculated size of 191,204 Dalton (Da). A Myc-epitope tagged full length VKIND protein, which was transiently expressed in NIH 3T3 fibroblasts, migrates with an apparent molecular weight of 205 kDa on a SDS-polyacrylamide gel (data not shown). Expression analysis of the *VKIND* gene in various tissues of an adult mouse by Northern hybridization revealed a very restricted expression pattern of the gene (Fig. 2). Strong expression could only be detected in brain. A very faint staining was also detectable in the RNA preparation from ovary. The *VKIND* mRNA migrated much slower on a formaldehyde agarose gel than the 28s subunit of the ribosomal RNA (4.7 kb) and had an estimated size of 7–8 kb.

To analyze the developmental time course of *VKIND* expression RNA in situ hybridizations were performed on sagital and transverse sections of mouse embryos. Especially the developing brain and the neural tube exhibited strong staining. At E14.5 *VKIND* was highly expressed in the mid- and hindbrain and only weakly in the forebrain, but during development the main expression shifted towards the telencephalon as seen at E17.5 (Fig. 3A, B). In the neural tube expression started at E10.5 at the ventro-lateral part where motoneurons are already positioned at this time (Fig. 3C). The area of expression expanded towards the dorsal part during development (Fig. 3C, F, I). The expression pattern of *VKIND* (Fig. 3C) in the developing neural tube at E10.5 partially overlaps with *islet1* as a marker for post mitotic motoneurons within the neural tube (Fig. 3D) (Appel et al., 1995) and *doublecortin* as a marker for early post mitotic migrating neurons (Fig. 3E) (Hannan et al., 1999). In contrast the developing DRG strongly stain for the *islet1* mRNA (Fig. 3D) while *VKIND* is not expressed in these cells at E10.5 (Fig. 3C). At E12.5 few cells in the DRG also stained positive for the *VKIND* mRNA (Fig. 3F). At E14.5 the entire neural tube except for the marginal layer was stained, but only in a speckled pattern indicative of cell type specific and presumably neuronal expression pattern (Fig. 3I). The expression of *VKIND* mRNA in the developing eye at E17,5 (Fig. 4A) very much coincides with the expression of *islet1* (Fig. 4B) and *doublecortin* (Fig. 4C). During development of the vertebrate eye *islet1* has been shown to be a marker for the ganglion cell layer (GCL) (Sakagami et al., 2003). This strongly indicates a very specific expression of *VKIND* in retinal ganglion cells (RGCs) in the developing eye. Expression of *VKIND* in the eye at E17,5 was found to be transient, since at erlier stages

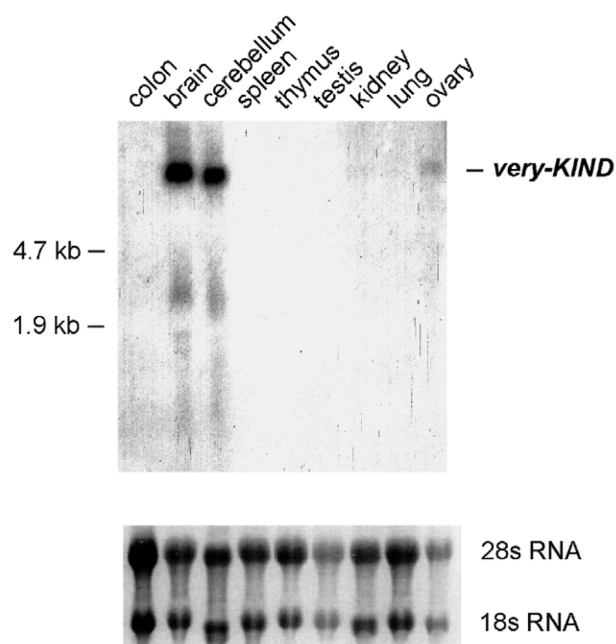


Fig. 2. *VKIND* mRNA expression. The expression of the *very-KIND* gene in different tissues of an adult mouse was analyzed by Northern hybridization. Total RNA preparations of the indicated tissues were separated on a formaldehyde-agarose gel and blotted onto a Duralon UV membrane. The *very-KIND* mRNA was detected with an (α-^{32}P)dCTP labelled mouse *very-KIND* cDNA fragment. The size of the ribosomal RNAs 4.7 and 1.9 kb (4.7, 1.9 kb) are indicated. The migration of the *very-KIND* mRNA is marked (*very-KIND*). As a loading control the ethidium bromide stained 28 and 18 s ribosomal RNAs are shown.

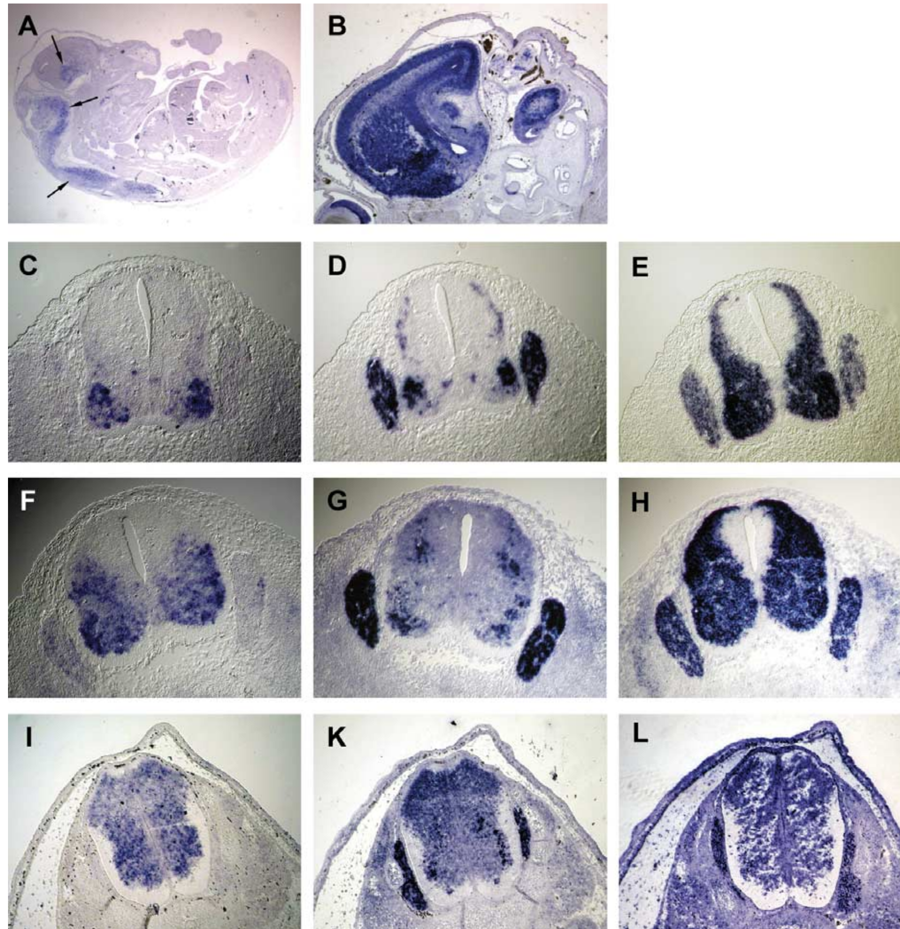


Fig. 3. Expression pattern of *VKIND* in the mouse embryo. (A) At E14.5 *VKIND* was expressed mainly in the mid- and hind-brain and the neural tube (arrows). (B) The expression in the neural tube persisted at E17.5, but additionally there was strong expression of *VKIND* in the telencephalon. (C, F, I) In the neural tube the expression of *VKIND* started at E11.5 (F) at the ventral edge and expanded dorsally during development (E12.5, F; E14.5, I). The expression of the neuronal markers *islet1* (D, G, K) and *doublecortin* (E, H, L) was shown for comparison.

of development and in post natal mice no *VKIND* expression could by detected.

In the adult brain the expression was most prominent in the cerebellum, but restricted to the granular and Purkinje cell layer (Fig. 5A, B). No staining was detected in the branching white matter of the cerebellum and in the molecular layer. The expression in Purkinje cells and the absence of transcripts in the white matter again suggests a preferential expression of the *VKIND* gene in neuronal cells. This is also supported by the punctuate expression of *VKIND* in large cells throughout most of the cerebral cortex (Fig. 5C).

The Very-KIND protein is a novel signalling protein specifically expressed in the nervous system of vertebrates. Although its homology to guanine nucleotide exchange factors of the Ras subfamily points towards a role in the activation of these GTPases, the enzymatic activity and specificity still has to be determined. Essential functions for RasGEFs in the nervous system have been described. RasGRF1 is a calcium dependent Ras specific guanine nucleotide exchange factor which mediates the signal transduction from the NMDA subtype of glutamate receptors at excitatory neuronal synapses to the Erk kinase pathway (Krapivinsky et al., 2003). The Rap1 specific exchange factor C3G is localized at signalling endosomes, which carry NGF retrograde signals from axon terminals to the cell body (Delcroix et al., 2003; Wu et al., 2001). A specific expression pattern as we have detected for the VKIND exchange factor, was also shown for the Rap specific exchange factor, MR-GEF. The expression of the *mr-gef* gene is regulated during differentiation of a specific subset of telencephalic neurons (Bithell et al., 2003). The RasGEFs are multi-domain proteins and encode next to their catalytic motifs structural domains, which regulate their activity and localization (Ehrhardt et al., 2002). Signals regulating the function of RasGEFs include phosphorylation of proteins and lipids, calcium fluxes, and the generation of cyclic nucleotides or diacylglycerol. The two KIND domains in the N-terminal part of VKIND may function in the integration of signals regulating the enzymatic activity or they might contribute to the attraction of substrates or mediation of the subcelluar localization.

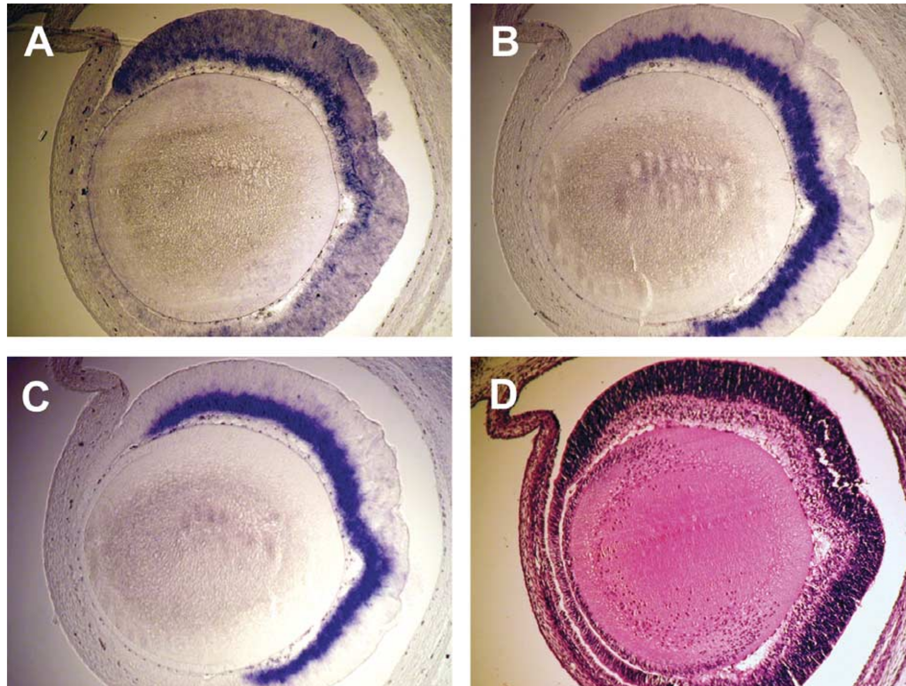


Fig. 4. Expression of the *VKIND* gene in the mouse embryonic eye. In the embryonic eye (E17.5) *VKIND* predominantly stained the future neuronal cell layers (A) similar to *islet1* (B) and *doublecortin* (C). Layers were defined according to Haematoxylin–Eosin (HE) staining (D).

In analogy to the other members of the KIND protein family Spir and PTPN13, VKIND may also have a function in cytoskeletal organization.

2. Experimental procedures

2.1. Cloning of the VKIND gene

A *very-KIND* cDNA fragment covering the complete coding region has been assembled from five cDNA fragments, which were generated by RT-PCR, employing a total RNA preparation from mouse cerebellum as a template. For the assembly the following restriction endonuclease recognition sites within the mouse *very-KIND* coding sequence have been used: *Xho* I (base 1007), *Bst*X I (base 2072), *Nhe* I (base 3174), *Xba* I (base 4044). The 5′ primer, which contains sequences encoding the Myc-epitope tag fused to the second codon of the mouse *very-KIND* gene, has the following sequence: 5′ GC TCT AGA AAG CTT GCC GCC GCC ATG GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG CAG GCC ATG GAC CCA GCC TCC 3′. The 3′ primer, which contains the translational stop codon and an *Eco*R I restriction endonuclease recognition site, has the following sequence: 5′ CG GAA TTC CTA CTG GAA TGT GGC CTT CAT 3′. The cDNA fragment has been assembled in the pSport1 cloning vector and inserted into the *Hin*d III and *Eco*R I sites of the pcDNA3 expression vector (pcDNA3-Myc-VKIND).

2.2. Northern blotting

Total RNA was isolated from organs of an adult C57BL/6 mouse by the guanidinium-thiocyanate-phenol-chloroform single-step extraction method (Stratagene RNA isolation kit). 10–20 μg of total RNA per organ were

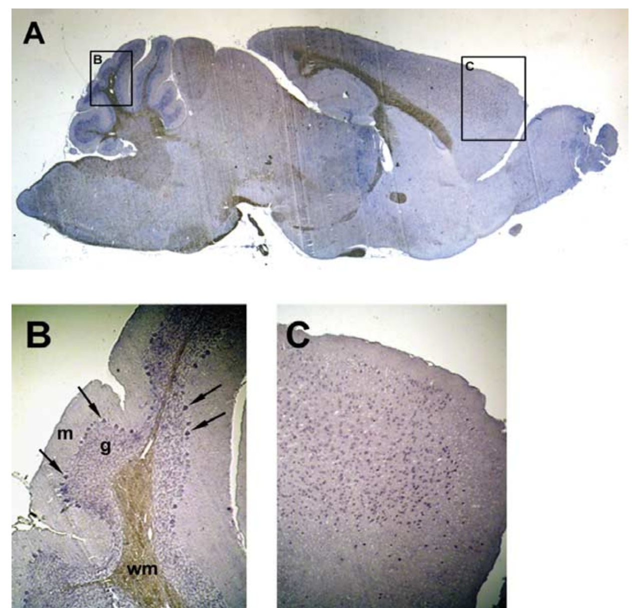


Fig. 5. Expression pattern of the *VKIND* gene in the adult mouse brain (A). In the adult brain *VKIND* was expressed in the granular and Purkinje cell layer of the cerebellum (arrows) (B) (Abr.: m molecular layer, g granular layer, wm white matter) and most likely in the neurons of the cerebral cortex (C).

separated on a formaldehyde-agarose gel and blotted onto a Duralon UV membrane (Stratagene). A 1 kb DNA fragment, encoding amino acids 2–335 of the mouse very-KIND protein, was labelled with (α-^{32}P)dCTP by random primed DNA labelling (Roche random primed labelling kit). The labelled DNA fragment was used as a probe for the Northern hybridization. The hybridization was performed as described before (Kerkhoff and Rapp, 1997).

2.3. In situ hybridizations

CD1 embryos or perfused mouse brain tissues were prepared for paraffine sections and hybridized with digoxigenin-labelled antisense riboprobes generated from plasmid templates as described previously (Leimeister et al., 1998). For the generation of the antisense riboprobes for mouse *very-KIND* (codon 2–126, accession: AJ580324.2), mouse *iselt1* (codon 129–349, accession: AJ132765.1) and mouse *doublecortin* (codon 124–360, accession: NM_010025.2) cDNA fragments were amplified by RT-PCR (Qiagen OneStep RT-PCR kit) from total brain RNA of an adult C57BL/6 mouse (*VKIND*) and from total RNA of a day 14.5 mouse embryo (*islet1*, *doublecortin*). The fragments were inserted into the pcDNA3 expression vector (*VKIND*, *Bam*H I/*Xba*I; *iselt1*, *Bam*H I/*Eco*R I; *doublecortin*, *Bam*H I/*Eco*R I). The following primer were used: *very-KIND*, 5′ CG GGATCC GCC GCC GCC ATG GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG CAG GCC ATG GAC CCA GCC TCC 3′, 5′ GC TCTAGA C CAG GGA GTA GAT GTG AGC CTC 3′; *islet1*, 5′ GC GGATCC TGC CGT GCA GAC CAC GAT GTG 3′, 5′ GC GAATTC TCA TGC CTC AAT AGG ACT GGC 3′; *doublecortin*, 5′ GC GGATCC AGC TAT GTC TGC TCC TCA GAC 3′, 5′ GC GAATTC TCA CAT GGA ATC GCC AAG TGA 3′.

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